

## **Historic, Archive Document**

Do not assume content reflects current scientific knowledge, policies, or practices.





United States  
Department of  
Agriculture

Marketing and  
Regulatory  
Programs

Animal and  
Plant Health  
Inspection  
Service

Plant Protection  
and Quarantine

# Karnal Bunt Emergency Program Manual

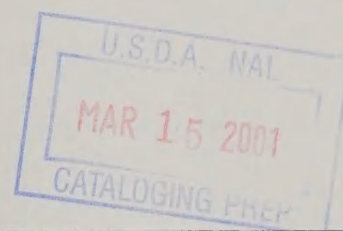




## CONTENTS

### Laboratory Procedures

	<u>Page</u>
<b>Introduction</b> .....	3.1
Purpose .....	3.1
Overview .....	3.1
<b>Setting Up the Laboratory Using Minimum Facility Requirements</b> .....	3.3
Introduction .....	3.3
Reception Area .....	3.3
Dirty Room .....	3.3
Clean Room .....	3.3
<b>Receiving Samples</b> .....	3.5
Introduction .....	3.5
Supplies .....	3.5
Sample Decontamination .....	3.5
Sample Log-In .....	3.7
Reserve Sample Storage .....	3.7
<b>Extracting Samples</b> .....	3.9
Introduction .....	3.9
Supplies .....	3.9
Preparing the Extraction Solution .....	3.11
Preparing Shear's Mounting Fluid .....	3.11
Preparing Extraction Flasks .....	3.13
Weighing and Measuring the Samples .....	3.13
Adding Extraction Solution .....	3.17
Extracting the Samples .....	3.18
Cleaning Up the Extraction Lab .....	3.21
<b>Preparing Slides</b> .....	3.23
Introduction .....	3.23
Supplies .....	3.23
Setting Up the Working Area .....	3.24
Identifying Sample Priority .....	3.25
Processing Sample Tubes .....	3.25
Preparing the Slides .....	3.25
Making Slide Labels and Filling Out the Data Sheet .....	3.27
Handling Spills .....	3.28
Cleaning Up .....	3.28



	<u>Page</u>
<b>Examining Slides for Karnal Bunt</b> . . . . .	3.29
Introduction . . . . .	3.29
Selecting a Slide . . . . .	3.29
Examining the Slide . . . . .	3.29
Identifying Karnal Bunt Teliospores . . . . .	3.29
Verifying Teliospores . . . . .	3.30
Counting Teliospores . . . . .	3.30
Reading Positive Slides . . . . .	3.30
Keeping Records . . . . .	3.31
Interpreting Laboratory Results . . . . .	3.32
 <b>Examining Wheat Kernels for Karnal Bunt</b> . . . . .	 3.33
Introduction . . . . .	3.33
Supplies . . . . .	3.33
Obtaining Samples . . . . .	3.34
Preparing the Workstation . . . . .	3.34
Identifying <i>Tilletia indica</i> . . . . .	3.35
Storing Positive Kernels . . . . .	3.36
Decontaminating Labware and Instruments . . . . .	3.36
 <b>Training Readers and Verifiers</b> . . . . .	 3.37
Introduction . . . . .	3.37
Training Readers . . . . .	3.37
Selecting and Training Verifiers . . . . .	3.38

## LABORATORY PROCEDURES

### Introduction

#### Purpose

The purpose of processing wheat or other crop samples in the laboratory is to isolate and to identify teliospores of Karnal bunt (KB), *Tilletia indica*, that may be present. The presence or absence of KB teliospores will determine whether we will classify a sample positive or negative for KB.

The technique of extracting and identifying teliospores by selective sieving was developed by Gary Peterson, United States Department of Agriculture, Agricultural Research Service (USDA, ARS), Ted Boratynski, USDA, Animal and Plant Health Inspection Service, Plant Protection and Quarantine (APHIS, PPQ), and Joel Floyd, USDA, APHIS, PPQ. Carefully following the procedures outlined in this section will allow recovery of at least 80 percent of the spores present in a grain/seed sample. **Be very careful to maintain the identity of the grain samples.** APHIS management will make important economic decisions that may be based on results obtained from a single sample.

#### Overview

This is an overview of the tasks involved with laboratory procedures:

1. Setting up the laboratory using minimum facility requirements.
2. Receiving samples.
3. Extracting samples.
4. Preparing slides.
5. Examining slides for Karnal bunt.
6. Examining wheat kernels for Karnal bunt.
7. Training readers and verifiers.

*Figure 3-1: Overview of Laboratory Procedures Tasks*



## LABORATORY PROCEDURES

### Setting Up the Laboratory Using Minimum Facility Requirements

#### Introduction

Whether laboratory personnel process a few samples per day or 100, the same minimum facility requirements are necessary. By meeting these requirements, each laboratory will have the flexibility to use its available resources, while still maintaining a national standard. The laboratory must have three basic areas:

- A reception area
- A dirty room
- A clean room

Combining functions performed in the dirty room and the clean room into one room is acceptable under the following conditions:

- Samples for processing come from a non-KB area.
- No positive samples have been in the laboratory.

Once a suspect positive sample is encountered, these rooms and functions need to be separate. Also, workers should always process a negative control wheat sample with each group of samples once any samples entering the laboratory test KB positive.

#### Reception Area

The reception area, a place for initial delivery of samples and decontamination of packaging, must be outside the laboratory facilities, whether literally outside or in a designated building, loading dock, or shed. This area can also be used for storing the remaining sample not used in the laboratory evaluation. The area must have access to running water.

#### Dirty Room

The dirty room is an area for opening dry grain samples and transferring or weighing samples. The primary concern is to prevent dry, loose teliospores from becoming airborne and contaminating other samples and the external surfaces of the extraction equipment. The minimum equipment required is a clean air station or biological containment hood, to prevent direct air movement and ventilation from this room into the clean room. Between each 50 g subsample weighed, workers must wipe down all working surfaces with detergent and 30 percent household bleach (5.25 percent sodium hypochlorite). Technicians must change gloves between samples and not wear contaminated garments into clean areas.

#### Clean Room

No sample should enter the clean room unless it is in liquid suspension. Workers moving from the dirty room into the clean room should have separate lab coats and preferably shoes or shoe covers in each area, which will remain in those areas. Activities performed in the clean room are sample extraction, slide preparation, extraction ware decontamination, and microscopy. Laboratory personnel must wipe down the extraction table surfaces between sets of samples. **Workers must clean up any spills before they dry and spray the area of the spill with 30 percent household bleach.** After extracting samples on trays, remove and decontaminate the trays.



## LABORATORY PROCEDURES

### Receiving Samples

#### Introduction

This subsection describes procedures used to receive grain/seed samples at approved laboratories for the analysis of *Tilletia indica*. Included are specific procedures for decontaminating the grain/seed samples before they enter the laboratory, and log-in procedures.

#### Supplies

Laboratory personnel will need the following supplies to receive the samples:

- Biohazard bags, 12" x 24"
- Bleach, household (sodium hypochlorite), 5.25 percent
- Cardboard boxes
- Chain-of-custody seals (if appropriate)
- Container to hold bleach solution
- Coveralls
- Dish pans, plastic, department store
- Garden hose with nozzle, hardware store
- Karnal bunt spore analysis worksheets, in-house designed
- Lab coats
- Labels
- Latex gloves
- Safety glasses
- Secure storage units for samples
- Shoe covers
- Spray bottle, 1-liter, hardware store
- Timer
- Utility cart

#### Sample Decontamina- tion

Samples usually arrive packed inside brown paper bags. If from the National Survey, the samples will be double-bagged, without subsamples. Sample bags from the Emergency Program may contain multiple subsamples consisting of 250-mL plastic bottles and/or plastic zip-seal bags of grain. When samples arrive at the laboratory, make sure to receive them at a designated check-in/decontamination area. This area must be physically separate from the laboratory facility where samples are processed. A wash basin and hose setup is necessary at this location. Use the following procedure to check in and decontaminate the samples before bringing them inside the laboratory. **Wear gloves, safety glasses, lab coat, coveralls, and shoe covers during this procedure.**

#### Step 1:

Check each sample against the accompanying paper work to ensure matching sample number. Check all seals (if used) and samples for integrity.

**Step 2:**

Decontaminate the sample containers, using **Table 3-1** as a guide:

**Table 3-1: Decontamination of Samples**

If the samples (or subsamples) are in:	Then:
Paper bags ( <b>not</b> containing subsamples)	<ol style="list-style-type: none"> <li>1. PLACE the sample bags inside new, clean paper bags.</li> <li>2. GO to <b>Step 3</b>.</li> </ol>
Plastic bottles	<ol style="list-style-type: none"> <li>1. REMOVE the bottles from the sample bag.</li> <li>2. TIGHTEN the lids to ensure a water tight seal.</li> <li>3. FILL the left-hand side of a wash basin with water.</li> <li>4. PLUNGE the bottles into the water.</li> <li>5. PLACE the bottles in the empty right-hand side of the wash basin.</li> <li>6. FILL a spray bottle with 30 percent bleach solution, prepared fresh daily.</li> <li>7. SPRAY the bottles with the bleach solution and ALLOW the solution to remain on the bottles for 15 minutes.</li> <li>8. After 15 minutes, RINSE the bottles with tap water from a garden hose.</li> <li>9. GO to <b>Step 3</b>.</li> </ol>
Plastic bags	<ol style="list-style-type: none"> <li>1. MAKE sure the bags are sealed tightly.</li> <li>2. FILL a spray bottle with 30 percent bleach solution, prepared fresh daily.</li> <li>3. SPRAY the bags with the bleach solution and ALLOW the solution to remain on the bags for 15 minutes.</li> <li>4. After 15 minutes, RINSE the bags with tap water from a garden hose.</li> <li>5. GO to <b>Step 3</b>.</li> </ol>

**Step 3:**

Take the samples into the sample receiving area of the laboratory for log-in. **Do not take any samples into the laboratory before decontaminating them.**

**Step 4:**

Store excess bottles and bags of grain in a locked storage unit. Sign and date any accidentally broken seals. Reseal the samples if necessary. Dispose of used gloves in a biohazard bag at the decontamination area.

**Sample Log-In** Accurate sample log-in is very important. Be very careful to maintain each sample's identity.

**Step 1:**

List the assigned sample number on the spore analysis worksheet, and affix a label with the laboratory number to the sample container. Place the samples on a utility cart after logging-in and labeling them. Continue logging the samples in and listing them on the spore analysis worksheet until reaching a maximum of 11 samples. Enter a "Quality Assurance (QA) Negative" laboratory control into the computer and list on the worksheet as well. Make sure to include on the cart a label for the control (for assembly in the weighing room). Use positive laboratory controls periodically (not less than once every tenth extraction set) to prove the laboratory's ability to identify positive samples.

**Step 2:**

If the samples are in bags instead of plastic bottles, place the labels on prepared Erlenmeyer flasks (see extraction procedure for preparation of flasks).

**Step 3:**

Label centrifuge tubes with laboratory sample numbers for each sample in each set. Place the tubes in a disposable tube rack supplied with the tubes, and label the rack with the set number. Place the samples (and flasks if applicable), worksheet, and centrifuge tubes on a utility cart, and deliver these items to the weighing area or the solution addition area.

**Reserve Sample Storage** Using approved sample journals, label sample bags and bottles with correct laboratory sample numbers. Group samples by set number, and place them in cardboard boxes in a designated location. Identify the boxes appropriately.



## LABORATORY PROCEDURES

### Extracting Samples

#### Introduction

This subsection describes procedures used at approved laboratories to extract teliospores of *Tilletia indica* from wheat or other crop samples. Included are specific procedures for preparation of extraction solution and preparation of Shear's mounting fluid. Also included are procedures used to prepare extraction flasks and measure out samples, and an alternative method for adding direct extraction solution to premeasured bottled samples. This subsection also details the extraction technique itself.

#### Supplies

Laboratory personnel will need the following reagents, equipment, and other items to process the samples:

##### Reagents:

- Bleach (5.25 percent sodium hypochlorite), household
- Citric acid monohydrate ( $C_6H_8O_7 \cdot 1H_2O$ )
- Deionized water
- Ethyl alcohol, 95 percent, anhydrous
- Glycerol
- Isopropyl alcohol, 70 percent
- Micro laboratory detergent
- Potassium acetate ( $KC_2H_3O_2$ )
- Sodium phosphate, dibasic, anhydrous ( $Na_2HPO_4$ )
- Tween 20

##### Suggested Equipment and Other Items:

- Aprons, rubber, 45" x 35"
- Bags, bio-hazard, 38" x 48"
- Bags, bio-hazard, 12" x 24"
- Balance, analytical
- Beakers, glass, 2-L and 1-L
- Beakers, 600 mL
- Bottle, glass, 1-L with ground glass stopper
- Bottle, spray, plastic, 1-L, hardware store
- Bottles, HDPE wide mouth, 250-mL, plastic
- Bottles, wash, 500-mL
- Box, broken glass disposal
- Bulb, pipette
- Carboy, 20-L with spigot
- Cart, glassware
- Cart, utility, stainless steel
- Cartridge, respirator (HEPA)
- Centrifuge, minimum 1,000 r/min required

**Suggested Equipment and Other Items (continued):**

- Cups, disposable, paper, 5-oz
- Cups, disposable, paper, 3-oz
- Cups, portion, paper, 1-oz
- Cylinders, graduated, 25-mL, 250-mL, 500-mL, 1-L
- Dissecting microscope, linen tester, or 10X magnifying glass
- Dust masks
- Flasks, Erlenmeyer, graduated, 500-mL
- Funnels, glass, 60-mm ID top x 13-mm OD stem
- Funnels, glass, 100-mm ID top, 18-mm OD stem
- Glasses, safety
- Gloves, gauntlet, sizes 9, 10, and 11
- Gloves, latex, surgical
- Gloves, nitrile, sizes 8, 9, 10, and 11
- Hood, biosafety with HEPA filter
- Hoods, fume
- Labels, 1" x 2<sup>5</sup>/<sub>8</sub>", 3 up x 10, Avery, 5160
- Liner, bench (substitute for plastic trays)
- Mixer, vortex, thermolyne
- Parafilm
- Pipette tips, 200-1000  $\mu$ L, for Eppendorf adjustable pipettor
- Pipettes, Mohr, 1-mL and 10-mL
- Pipettor, adjustable, Eppendorf, 100-1000  $\mu$ L
- Pitcher, 3000-mL
- Rack, test tube
- Scoop, lab, stainless steel
- Shaker, orbital, thermolyne
- Sieve, nylon, 20  $\mu$ m pore with square openings, 3" ID x 50 mm high, USDA\*
- Sieve, nylon, 53  $\mu$ m pore with square openings, 3" ID x 50 mm high, USDA\*
- Stir bars, magnetic, 1<sup>1</sup>/<sub>2</sub>" x 1/2"
- Stirrer, magnetic
- Tape, lab, blue, 1"
- Tape, lab, red, 1/2"
- Tape, yellow stretchy, lab safety
- Terri-wipes
- Timers
- Trays, plastic, 18" x 12" x 1/2" deep (substitute for bench liners)
- Tubes, centrifuge, disposable, plastic, conical, 15-mL
- Tubs, disinfectant
- Tubs, self-draining and holding

\*See Appendix 4 for construction of the sieves.

### Preparing the Extraction Solution

Prepare the extraction solution in a separate, clean room. **Never bring grain samples into this room.**

#### Step 1:

Wearing safety glasses and latex gloves, fill a 20-L carboy with nearly 18 L of deionized water.

#### Step 2:

Using a graduated cylinder, add 12 mL Tween 20 detergent to the carboy. Rinse the cylinder several times with deionized water into the carboy.

#### Step 3:

Fill the carboy to the 18-L mark. Place a strip of red lab tape at the 18-L mark and label as Tween 20 solution. Cap tightly and roll the carboy on a utility cart to make sure that the Tween is well dispersed in the water.

### Preparing Shear's Mounting Fluid

After centrifuging aqueous extracts of *Tilletia indica* and decanting the supernatant, re-suspending the remaining pellet in mounting fluid is necessary. Shear's mounting fluid, the standard mounting medium for smut fungi, provides excellent buffering capacity. The fluid maintains the structural integrity of the KB spores, while limiting evaporation. Additionally, the glycerol in the Shear's fluid maintains a refractive index of 1.47 at 27 °C, which allows the effective transmission of light by the compound microscope. Shear's mounting fluid preserves sample extracts for future microscopic evaluation.

*Prepare one liter of 0.2 M Na<sub>2</sub>HPO<sub>4</sub> solution by following these steps:*

#### Step 1:

Wearing safety glasses and latex gloves, weigh 38.39 g of anhydrous Na<sub>2</sub>HPO<sub>4</sub> into a tared 2-L beaker on a 3 decimal balance.

#### Step 2:

Using a graduated cylinder, add 1 L of deionized water to the beaker and stir with a magnetic stirring bar until all granules are dissolved.

**Step 3:**

Using a funnel, transfer the solution to a 1-L glass stoppered bottle and label as 0.2 M  $\text{Na}_2\text{HPO}_4$  with the date of preparation and your initials.

*Prepare 1 L of 0.1 M citric acid solution by following these steps:*

**Step 1:**

Weigh 21.01 g of citric acid monohydrate into a tared 2-L beaker on a 3 decimal balance.

**Step 2:**

Using a graduated cylinder, add 1 L of deionized water to the beaker and stir until all granules are dissolved.

**Step 3:**

Using a funnel, transfer the solution to a 1-L glass stoppered bottle and label as 0.1 M citric acid solution with the date of preparation and your initials.

*Prepare 600 mL of Shear's Mounting Fluid by following these steps:*

**Step 1:**

Using a pipette, transfer 19.45 mL of 0.2 M  $\text{Na}_2\text{HPO}_4$  into a 1-L beaker.

**Step 2:**

Using a 1-mL pipette, transfer 0.55 mL of 0.1 M citric acid solution into the same beaker.

**Step 3:**

Add 6 g of potassium acetate and 280 mL of deionized water to the beaker.

**Step 4:**

Stir with a magnetic stirring bar until all solids are dissolved and the solution is uniform in appearance.

**Step 5:**

Continue stirring and add 120 mL of glycerol and 180 mL of 95 percent ethyl alcohol to the beaker.

**Step 6:**

After the solution is uniform in appearance, use a funnel to transfer to a 1-L glass stoppered bottle. Label as Shear's Mounting Fluid, and include the date of preparation and your initials.

Clean all glassware with lab detergent and rinse with tap water followed by deionized water. Place pipettes in the pipette washer with tips up.

**Preparing  
Extraction  
Flasks**

Prepare and label an Erlenmeyer flask containing extraction solution for each sample that requires weighing or measuring out before extraction. Carry out the following procedure in the room designated for preparing extraction solution:

**Step 1:**

Wearing safety glasses and latex gloves, fill 500-mL flasks to the 100 mL mark with extraction solution directly from the 20-L carboy. Place two 3-oz disposable paper cups over the mouth of each flask immediately after adding the solution.

**Step 2:**

Label one flask for each sample in the set to be extracted. Place the flasks for that set on the top shelf of a utility cart in the order listed on the worksheet and move it to the sample weighing area.

**Step 3:**

Place the spore analysis worksheet and labeled centrifuge tubes on the top shelf of the cart. Place the samples in order from left to right on the second shelf of the cart and take the cart to the room designated for measuring out the samples.

**Weighing and  
Measuring the  
Samples**

Take samples that require weighing or measuring out to the sample weighing area. Carry out this procedure in a biosafety hood fitted with a HEPA filter. Generally, the only samples of this type are bin sweepings or other "dirty" samples that contain mostly dirt and chaff and very little grain. Measure most other samples by volume rather than by weight. Consult your supervisor if weighing or measuring unusual sample matrices or sample types is necessary. **Wear safety glasses, latex gloves, lab coat, overalls, and shoe covers throughout this procedure.**

*Sanitizing the biosafety hood*

Before weighing or measuring any samples, first sanitize the biosafety hood thoroughly.

**Step 1:**

Turn on power to the hood, light, and balance.

**Step 2:**

Thoroughly sanitize the interior of the hood (including walls, glass door, and ceiling) by spraying with 30 percent bleach solution. This also includes the balance and the area under the balance. Let the bleach stand for 15 minutes.

**Step 3:**

Spray the hood again with the bleach solution and wipe dry with terry wipes. Dispose of used terry wipes in the biohazard bag.

**Step 4:**

Spray the entire hood with 70 percent isopropyl alcohol and wipe dry. Doing this will help to clean bleach residues from hood surfaces, particularly the window. Dispose of used terry wipes in the biohazard bag.

*Weighing the samples*

For samples that are very dirty and contain very little sample by weight, measure them out by weighing a 2.0 g portion of sample on an analytical balance. Wear a dust mask when handling dirty and dusty samples.

**Step 1:**

From the cart containing the sample set to be weighed, take a sample and make sure that the sample number and the number on the 500-mL flask of extraction solution are identical. Place the sample, the corresponding flask, and a 4x4 square of parafilm in the hood (include the two 3-oz paper cups supplied with the flask). Pull down the hood door to allow only enough space to move sample flasks in and out of the hood.

**Step 2:**

Put on a new pair of latex gloves; place one of the two paper cups on the balance and tare it.

**Step 3:**

Mix the sample well by inverting and rolling thoroughly. Open the sample container and use one paper cup to scoop out  $2.0 \text{ g} \pm 0.1 \text{ g}$  of sample into the cup on the balance. Transfer back to the sample container the excess sample that has not touched the hood or balance.

**Step 4:**

Transfer the contents of the paper cup to a 500-mL bottle or flask and seal the opening with parafilm. Pull down on the parafilm to make an airtight seal.

**Step 5:**

Close the sample container and place the two cups and the paper backing from the parafilm in the palm of one gloved hand and fold that glove over them. Now place this glove in the palm of the other hand and repeat. Dispose of the used materials in the biohazard bag.

**Step 6:**

Remove the sample and the flask from the hood, and place the flask back on the cart. Place the sample in a cardboard box for storage.

**Step 7:**

For additional samples, repeat **Steps 1 through 6** using new gloves for each sample. Deliver the cart to the Extraction lab after weighing all samples. Return sample bags to the storage area.

**Step 8:**

Always maintain cleanliness of the weighing area. If any sample falls on the balance or the hood floor, **do not** place in the flask or return to the sample container. Dispose of the sample with the gloves and cups at the end of the weighing cycle. If any sample falls on the hood or balance, sanitize the area of the spill before weighing the next sample. Wipe up the spilled sample and dispose of it in a biohazard bag. Spray the area where the sample fell and the surrounding area with the bleach solution. Wipe up the bleach solution and dispose of the dirty gloves and terry wipes in the biohazard bag.

**Step 9:**

Resanitize the hood and balance after each sample.

*Measuring the samples*

Measure by volume these types of grain samples:

- Samples that do not arrive premeasured in plastic bottles
- Samples that require additional analyses after previous extraction of all bottles

Measure these samples into Erlenmeyer flasks or into new, empty plastic bottles if available.

**Step 1:**

The procedure is the same as for weighing samples in the previous section, except that this procedure involves measuring the samples by scooping three 1-oz cupfuls into a flask or bottle (may be different for other types of crops). Use the paper portion cups for this purpose, and then dispose of them in the biohazard bag.

**Step 2:**

When measuring the samples into plastic bottles, take them by cart to add extraction solution before taking them to the Extraction lab. See **Adding Extraction Solution** below.

**Step 3:**

Also, measure control wheat into clean plastic bottles using the above procedure. Seal these bottles of control wheat and store them in the solution addition room for later use in extraction sets as needed.

*Preparing the biosafety hood for the next shift***Step 1:**

When finished weighing or measuring samples for the day or at the end of a shift, resanitize the hood as described in the subsection *Sanitizing the biosafety hood*.

**Step 2:**

Turn off balance and hood light. Leave the exhaust blower on.

**Step 3:**

Restock any supplies that are running low. Replace the biohazard bag if it is full. Tie it shut with lab tape and place the bag inside the appropriate biohazard storage area.

### **Adding Extraction Solution**

When an extraction set consists of premeasured samples in plastic bottles, add the extraction solution to the bottles in the biosafety hood fitted with a HEPA filter in the solution addition room. Take only decontaminated sample bottles into this room. Take bagged samples that require measuring to the weighing area. **Wear safety glasses and latex gloves throughout this procedure.**

#### **Step 1:**

Turn on power to the biosafety hood and sanitize the hood as described in the subsection *Sanitizing the biosafety hood*. Pull down the hood door to allow only enough space to move sample bottles in and out of the hood.

#### **Step 2:**

Before adding solution to any of the bottles, first check to make sure that each sample on the utility cart has a matching sample number listed on the worksheet. The bottles or flasks should also appear to contain approximately 50 g of sample for wheat; other crops may be different. If any bottle appears to contain too much or too little sample, call it to the attention of your supervisor. If your supervisor approves rejecting the sample for improper volume, measure a new sample from the bag of grain retained in storage.

#### **Step 3:**

Place one sample bottle in the hood and put on a new pair of latex gloves. Add extraction solution from the carboy on the counter outside the hood to a clean, disposable paper cup. Fill to a level equal to the mark on the reference cup next to the carboy. This should be approximately 100 mL of solution. Place the cup of solution in the hood and open the sample bottle. Add the solution to the bottled sample. Tighten the bottle cap and place the sample back on the cart. Place the cup in the palm of one gloved hand and fold that glove over it. Now place this glove in the palm of the other hand and repeat. Dispose of the gloves and cup in the biohazard bag. Repeat this procedure for each sample on the cart, using a new pair of gloves and cup for each sample.

#### **Step 4:**

Follow the procedures described in the subsection *Preparing the biosafety hood for the next shift*.

**Extracting the  
Samples**

The extraction lab must have sufficient counter space (approximately 15 ft) to carry out the extractions. The lab must also have a shaker table, a chemical fume hood for bleach baths, and sinks to rinse glassware. A separate counter top is necessary to support the centrifuge station.

Extract the samples in the Extraction lab, always wearing safety glasses. As an added precaution, wear protective clothing such as a rubber apron and heavy rubber gloves when decontaminating glassware with bleach solution. Wear latex gloves when extracting samples of grain treated with fungicide.

*Setting up for extraction***Step 1:**

Place the laboratory bench liner on both sides of the extraction bench top with the plastic side down. To prevent confusion when processing multiple samples, use lab tape to mark out up to 12 lanes, approximately 30 cm wide on each side of the bench. Another approved method is to use individual plastic trays (18" x 12" x ½") for each sample. Plastic trays will contain spills, and tubes cannot roll into the wrong lane. The trays also work well at smaller labs that do not have room to set up multiple lanes.

**Step 2:**

Place one 600-mL beaker toward the rear of each lane and set a 20  $\mu$ m pore nylon sieve on top of it. Place another 600-mL beaker in front of the first beaker and set a 53- $\mu$ m pore nylon sieve on top of that beaker. Lay out as many beaker and sieve sets as there are samples to extract, and place centrifuge racks between each set. Place small glass funnels (60 mm ID) in the center of the bench, along with pitchers and wash bottles containing tap water. When using the plastic tray option, keep all the extraction equipment for one sample in that sample's tray.

**Step 3:**

Prepare bleach baths by filling large disinfectant tubs inside fume hoods with 30 percent bleach solution. Prepare 2-4 bleach baths, depending on expected sample volume for the shift.

### *Extracting the samples*

#### **Step 1:**

When sample bottles or flasks containing extraction solution arrive on the utility cart, transfer them to an orbital shaker on the end of the extraction bench. Secure the containers in the shaker so that the fit is appropriate for the type of container used. Shake samples for 3 minutes at 200 r/min. Do not allow samples to remain in the extraction solution for longer than 30 minutes due to release of starch granules. While samples are shaking, remove the centrifuge tubes that arrived with the samples from the disposable rack and place one per lane on the bench top. Place the disposable centrifuge tube rack and worksheet in the center of the bench. Sanitize the utility cart with 30 percent bleach solution before returning to the sample receiving area.

#### **Step 2:**

After shaking the samples, place each in the lane with the tube that corresponds to that sample. Double check to make absolutely sure that tubes and sample bottles have matching sample numbers. Using the plastic tray option helps to eliminate errors.

#### **Step 3:**

For each sample, invert the bottle or swirl the flask to re-suspend the grain and debris. Immediately pour the contents into the 53- $\mu$ m sieve, collecting the filtrate in the 600-mL beaker. Add an additional 100 mL of water to the container, swirl or recap and shake the bottle, and then pour into the sieve to rinse the grain. Take care to avoid splashing the sample solution during sieving procedures. Reextract samples if cross-contamination occurs. Rinse a minimum of twice, tilting the sieve on top of the beaker to aid draining. Place the empty extraction container on the glassware cart.

#### **Step 4:**

After the sieve has drained, discard the grain into a small biohazard bag, and place the used sieve on a glassware cart. Pour the filtrate from the beaker into the 20- $\mu$ m sieve, collecting the filtrate in the second 600-mL beaker. Add an additional 100 mL of water to the first beaker, swirl, and pour through the sieve. Use a wash bottle to rinse down the side walls of the beaker, and pour this rinsing into the sieve as well. Rinse a minimum of twice. Place the used beaker on the glassware cart.

#### **Step 5:**

Once the 20- $\mu$ m sieve has drained, tilt the sieve on top of the beaker and use the wash bottle to rinse down the sides and upper portion of the nylon screen. Repeat this rinse. Debris will pool in the lower portion of the sieve.

**Step 6:**

Open the centrifuge tube and place it upright in the centrifuge tube rack. Place a small glass funnel in the tube and use a wash bottle to rinse debris from the 20- $\mu$ m sieve into the tube. Rinse the funnel into the tube as well. To assure that all tubes have the same amount of liquid, fill the tubes with liquid to just below the screw cap threads. Recap the tube and return to the disposable centrifuge rack. Pour the extraction filtrate into a 20-L carboy containing  $\frac{1}{2}$  gallon of bleach. Place filtrate from samples treated with fungicide in a separate carboy. Place used glassware and sieve on the glassware cart.

**Step 7:**

Repeat **Steps 3 through 6** for each sample in the extraction set. If extracting multiple samples becomes necessary, processing all samples through one step before proceeding to the next step is most efficient. **Be sure to remain undistracted while doing this.** Take care not to mix samples or confuse the extraction steps. The plastic trays are effective in keeping samples' equipment and information together.

**Step 8:**

In case of a sample spill, notify your supervisor to decide if a second extraction of the sample is necessary. If so, discard the sample and show the result for that sample as "L" on the worksheet with your initials and date. Record all lab accidents in the lab notebook kept at the centrifuge station. If a sample spills on the bench liner, replace it with a clean liner. If any sample contacts the bench surface, spray a 30 percent bleach solution on the area to decontaminate it, and wipe with a clean towel. Spills are easier to handle with the plastic tray option.

**Step 9:**

Immediately transfer all used glassware and sieves from the glassware cart to a bleach bath and soak them for 15 minutes. Do not allow sieves to remain in the bleach bath for more than 15 minutes, as this will rapidly degrade the nylon screens. Rinse glassware and sieves thoroughly with tap water. Hang glassware on a rack to drain and place sieves in self-draining plastic totes. Discard plastic sample bottles after decontaminating them in the bleach bath.

**Step 10:**

After preparing all of the tubes for the set, take them to the centrifuge station and centrifuge at 1,000 r/min for 3 minutes. Pour off the supernatant and re-suspend the pellet by pipetting 500  $\mu$ l of Shear's mounting fluid into the tube. Recap and vortex a few seconds to mix the pellet with the Shear's fluid. Record the set information in the centrifuge log book before delivering the centrifuge tubes and worksheet to the microscope area.

## **Cleaning Up the Extraction Lab**

Clean up the Extraction lab by following these steps:

### **Step 1:**

At least once per day, soak the sieves in warm soapy water for 30–60 minutes to remove trapped particles and protein residues. This soaking is critical to maintain the proper size separation and flow rates of the sieves. When used on a daily basis, inspect the sieves daily for damage using a stereo microscope, linen tester, or 10X magnifying glass. Discard damaged sieves. Replace **all** sieves at least once a month.

### **Step 2:**

At the end of each shift, remove the bench liner and discard in a large biohazard bag along with the small bags of extracted grain and other potentially contaminated materials. Tie these bags shut with lab tape. Place in red biohazard containers in a designated hazardous waste area. When using plastic trays, removing and discarding bench liners is not necessary.

### **Step 3:**

Pour the bleached sample filtrate from the carboys down a sink drain at the end of each shift, or when the carboy is full. After filling any carboys containing filtrate from seed treated with fungicide during the shift, pour the contents into a labeled 55-gallon polyethylene drum in a designated hazardous waste area.

### **Step 4:**

Spray all bench tops, carts, and other potentially contaminated surfaces with 30 percent bleach solution. Wipe all these surfaces down at the end of each shift. Sweep and mop the floor in the extraction room with bleach water at the end of each shift as well.

### **Step 5:**

Soak all pitchers and wash bottles in 30 percent bleach solution for at least 15 minutes at the end of each shift. Take care to rinse these items thoroughly before using them again.



## LABORATORY PROCEDURES

### Preparing Slides

#### Introduction

This subsection describes procedures used to prepare sample slides for microscopic identification of KB teliospores. Included are specifications for lab garments and set-up of the working area. The subsection also identifies priorities in the processing of samples. Topics include receiving sample tubes, preparing slides, filling out slide labels and data sheets, preparing quality assurance slides, handling spills, and cleaning up. The slide preparation area should be separate from other sample processing areas.

#### Supplies

Laboratory personnel will need the following reagents, equipment, and other items to prepare the slides:

##### Reagents:

- Bleach (5.25 percent sodium hypochlorite), household
- Cytoseal 60, low viscosity
- Shear's fluid

##### Suggested Equipment and Other Items:

- Applicators, cotton-tipped
- Bags, bio-hazard 38" x 48"
- Bags, bio-hazard 12" x 24"
- Beakers, 150-mL, 250-mL, and 400-mL
- Bottles, plastic, narrow mouth
- Bottles, plastic, spray
- Box, microscope slide
- Boxes, glass disposal, floor
- Bulbs, rubber pipette
- Cabinet, storage
- Caps, bouffant, 21"
- Centrifuge, minimum 1,000 r/min required
- Cleanser, skin, antimicrobial
- Coats, lab, polyethylene, medium, large, and extra large
- Cover slips, microscope, 22 x 50 mm
- Flasks, Erlenmeyer, 500-mL and 1,000-mL
- Fume absorber
- Gloves, latex, medium and large
- Graduate, transfer pipettes, polyethylene, 5-mL
- Labels, multi purpose, white, 5/8" x 7/8"
- Petroleum jelly (100 percent)
- Pipettes, Pasteur, disposable, 9-inch
- Pipettes, transfer, Padl-Pet 0.5-mL
- Scissors
- Slide warmer
- Slides, microscope, 3" x 1"
- Teri Reinforced Towels
- Tray, slide, aluminum or plastic

Store all supplies in a designated KB cabinet. Designate a clean area specifically for slide preparation. Clean all surfaces in this area with a 30 percent bleach solution before setting up the work area.

### Setting-Up the Working Area

Prepare the slides under a fume extractor or canopied laminar flow hood to minimize exposure to chemicals. Keep a supply of Shear's solution on hand. During the process of slide making, Shear's solution is necessary to dilute dense samples or to add liquid under the cover slip as needed.

#### Step 1:

Before making any slides, disinfect surfaces with 30 percent bleach solution from a spray bottle.

#### Step 2:

Fill plastic bottles with Cytoseal and Shear's solution. Label the bottles.

#### Step 3:

Label four sample tubes as blanks for balancing the centrifuge; add 2 mL of water in each tube.

#### Step 4:

Use an empty bleach bottle to fill half way with 30 percent bleach solution for disposal of Pasteur pipettes.

#### Step 5:

Label a large plastic screw-cap bottle with "Quarantine Material." Add 200 mL of 30 percent bleach. Place the bottle by the centrifuge. Use this bottle to decant supernatant from centrifuged samples.

#### Step 6:

Use new slides, cover slips, and pipettes for each sample. Check supplies and restock if needed. Prepare the plastic pipettes for Cytoseal solution by cutting the tips at a 45° angle using a scissors. Place plastic pipettes, Pasteur pipettes, and padl-pets in separate, clean beakers.

**Step 7:**

Under the extraction hood place one reinforced towel. On top of the towel, place a colored sheet of paper (throw away between samples). Turn on the slide warmer to approximately 58 °C. To avoid unnecessary air flow over the work area, do **not** turn on the fume extractor until ready to prepare the slides.

**Identifying  
Sample  
Priority**

Sample processing priority will vary by region. Laboratory managers will establish sample priorities based on program needs. Contact the laboratory manager if sample priority is in question.

**Processing  
Sample Tubes**

**Step 1:**

Count the tubes and data entries on the sheet. Make sure that the sample number matches the data sheet number, along with the set or box number. Report discrepancies to the laboratory manager.

**Step 2:**

Centrifuge samples at 1,000 r/min for 3 minutes. Adjust blank sample tubes to balance the centrifuge. Decant supernatant into the "Quarantine Material" bottle. Do **not** pour off the supernatant of large or dense sample pellets.

**Preparing the  
Slides**

**Step 1:**

Use a new piece of paper, pipettes, gloves, slide(s) and cover slip(s) for each sample. Use clean pipette bulbs for each sample if not using cotton-plugged Pasteur pipettes. Move the sample tube to be prepared to the front of the rack. Put on new gloves. Use a Pasteur pipette with a pipette bulb to extract the pellet at the bottom of the tube. Squeeze the pipette bulb before inserting into the tube. Slowly release the pressure on the pipette bulb to extract the pellet. Avoid extracting excess supernatant to reduce the number of slides required per sample pellet. However, transferring the entire pellet to slides is important.

**Step 2:**

When processing dense, muddy, or thick samples, introduce the Pasteur pipette into the sample tube and homogenize the sample by pressing the pipette bulb several times. Homogenize the sample between each slide. Maintain pressure on the pipette bulb and remove the pipette from the tube. Hold the pipette at a 45° angle 2-3 inches above the slide and expel two or three small drops onto the slide depending on the sample density. For dense samples, dispense two drops of sample onto the slide and using a plastic pipette, place a drop of Shear's fluid in the middle. Doing this will help produce a nonlayered, uniform slide.

**Step 3:**

Use the end of the Pasteur pipette to spread the sample to a small rectangle. Avoid placing excess sample on the slide to eliminate seepage, or preparing a slide that will be too dense to read easily.

**Step 4:**

Return the tube with Pasteur pipette to the sample rack. Place a cover slip over the drops on the slide. Do this by holding the cover slip above the slide at a 45° angle and then touching the drop nearest the edge of the slide with the edge of the cover slip. A padl-pet pipette may help to move the opposite side of the cover slip down slowly until it touches the slide. This may help to reduce air under the cover slip. Remove air bubbles only by tapping lightly on the cover slip. Do **not** lift the cover slip to remove air bubbles.

**Step 5:**

Leave a ¼ inch margin between the cover slip and the slide edge opposite the label. Place the Pasteur pipette in 30 percent bleach solution. Cap the sample tube and discard the tube only after making the label.

**Step 6:**

Dispose of the padl-pet pipette in a biohazard bag. Seal the cover slip to the slide after preparing all of the slide(s) from a sample. Using a trimmed plastic pipette, extract the needed amount of Cytoseal. Seal the long sides of the cover slip, then the narrow sides to the slide. Release the Cytoseal slowly, running the pipette along the edge of the cover slip. Avoid making a pass over an area more than once, but inspect the perimeter of the cover slip for missed or poorly sealed areas. Touch up with Cytoseal as needed. Discard excess Cytoseal from the plastic pipette into a plastic screw cap bottle for disposal. Keep containers of Cytoseal and Shear's fluid closed when not in use. Remove your gloves and discard in the biohazard bag.

**Making Slide  
Labels and  
Filling Out the  
Data Sheet**

Make slide labels only for the current sample. **Never** use correction fluid on slide labels; make a new label.

**Step 1:**

Prepare the label from top to bottom using a permanent ink pen as follows:

**Table 3-2: Preparation of Slide Labels**

Description	Slide Label (Example)
Sample Code	CG-96-172
Number of slides prepared	1/3, 2/3, 3/3, etc.
Box or Set Number	Box 552
Person who read the slide	Reader Initials:
Authorized person who verified the positive finding of <i>Tilletia indica</i> or person who completed negative reread	Ver. Initials:
Date the sample was read	Date:

**Step 2:**

Affix the label(s) to the slide(s). Record the number of slide(s) prepared for the sample in the comment section of the data sheet.

**Step 3:**

Keeping the slide horizontal, place it on the warmer for 5 to 10 minutes. Transfer slides to a slide tray. Repeat the slide preparation for each sample in the rack.

**Step 4:**

After completing a data sheet of samples, count the number of slides prepared and compare it with the total listed on the data sheet. Initial "Slide Preparer" section on the data sheet. Record completion time on the lower right-hand corner of the data sheet. Use a separate log sheet to record the slide maker and number of slides made for an entire set of samples.

**Step 5:**

Place the tray and data sheet on the counter marked for reading. After readers read the slides and record the results on the data sheet, they will file the slides in a case by box or set number.

**Handling  
Spills**

Cover all spills of supernatant or sample with 30 percent bleach solution and a reinforced towel. Let soak for 15 minutes. Wipe up the bleach and rinse with water. Discard towels in a biohazard bag.

**Cleaning Up**

At the end of your shift, discard Cytoseal and Shear's solution in a biohazard bag along with the reinforced towel used under the extraction hood. Rinse pipette bulbs with 30 percent bleach solution for 15 minutes and set to dry. Tightly close the Pasteur pipette waste bottle, shake it a few times, and place it horizontally in the sink overnight. Turn off the extraction hood and slide warmer.

## LABORATORY PROCEDURES

### Examining Slides for Karnal Bunt

#### Introduction

This subsection describes procedures used for the microscopic identification of teliospores that may be *Tilletia indica*. A compound microscope is necessary to examine the slides. Included are procedures for selecting, examining, and identifying KB. The subsection also includes procedures for handling positive slides, negative slides, and record keeping. Microscopic identification requires a room with sufficient counter top and chair space to hold multiple microscope stations.

#### Selecting a Slide

Select a slide based on the priority established by the laboratory manager. Before taking a slide, place your initials in the "Slide Taken By" box and record the slide number in the "Slide" box of the TILLETIA INDICA SPORE ANALYSIS WORKSHEET (see **Appendix 1** for an example of a completed worksheet).

#### Examining the Slide

Examine the slide using the 10X objective and 10X ocular. Begin at one corner of the cover slip, including the area on the edge of the cover slip containing slide sealant. Scan the entire slide, either vertically or horizontally, left to right or right to left. Be consistent in your scanning procedure and be aware that the image is reversed. Take care to remember your direction if interrupted during a scan. A simple method is to place a pen or pencil on the table pointed in the direction of the scan at the time of interruption.

#### Identifying KB Teliospores

After carefully examining the *Tilletia indica* reference slides, and recognizing the variability in mature and immature spores, look for similar objects. Refer also to the laminated color illustration titled **Karnal Bunt Teliospores, *Tilletia indica* Mitra**, in **Appendix 1**. Upon finding a suspect spore, change the objective to 40X and look for the following characteristics:

##### Mature Teliospores:

- Spherical shape with an average diameter of 39 $\mu$ m and a range of 24–47 $\mu$ m. Atypical spores that are broken and/or irregularly shaped may be present.
- Color light brown to near black
- Smooth clear or yellowish sheath enveloping the spore that is visible when focusing on cross section of the spore
- Distinctive truncate projections that are visible on the circumference of the spore when focusing through a cross section and dark brown spots in the middle of the spore when focusing on the surface of the spore

**Immature Teliospores Combined With Mature Teliospores:**

- Similar shape but often smaller than mature teliospores
- Color yellow to lighter brown
- More distinct, finer ornamentation than mature teliospores

Any of the above characteristics will suggest a suspect find of *Tilletia indica* teliospores.

**Verifying  
Teliospores**

The appropriate authority for identification must verify the first *T. indica* teliospore found on each slide. The verifier will print his or her initials on the slide label. A trained coworker must verify the next four *T. indica* teliospores.

**Counting  
Teliospores**

When counting *T. indica* teliospores, including immatures, one of the first five teliospores must measure  $\geq 33 \mu\text{m}$  in diameter. Count only spores  $\geq 33 \mu\text{m}$ , and record the data on the TILLETIA INDICA SPORE ANALYSIS WORKSHEET. The verifier will record in the comments section spores  $< 33 \mu\text{m}$ . When finding only suspect spores smaller than  $33 \mu\text{m}$  in diameter, show this with an asterisk (\*) in the “# of spores” section of the worksheet. The asterisk will show that a retest will be necessary. The identifier should note in the comments section of the worksheet that the spore size is less than  $33 \mu\text{m}$ . Never measure across a crack in the spore.

Be certain also to count *T. indica* teliospores found under the slide sealant, on the edge of the cover slip. Circle the position of one “good” spore using a permanent marker on the bottom of the slide.

**Reading  
Positive Slides**

Use **Table 3-3** to count whole teliospores:

**Table 3-3: Counting Whole *T. indica* Teliospores**

If the number of teliospores you find is:	Then:
More than 30	Use the guide in Appendix 3, titled <b>Estimating the Number of <i>Tilletia indica</i> Teliospores in Heavily Infested Samples</b> , developed by Gary Peterson.
30 or less	Count all spores on the slide. Make marks on a piece of paper or use a mechanical counter to count spores.

Use **Table 3-4** to count fragments of teliospores. Fifty percent or more of the teliospore must be intact to allow a definitive measurement of the maximum diameter of the teliospore.

**Table 3-4: Counting Fragments of *T. indica* Teliospores**

If:	Then:
You find only fragments of less than 50 percent of the teliospore	Enter an asterisk (*) in the “# of spores” section of the worksheet, suggesting the order for a retest. The identifier should also note on the worksheet that it is a fragment.
Any of the first five spores on a slide are fragments of <i>T. indica</i> teliospores	An identifier must verify all fragments.
You find fragments of <i>T. indica</i> teliospores after the first five spores	Count if most of the spore is present. Having these fragments verified by an identifier is <b>not</b> necessary.

If multiple tests from the same sample result in one positive test, read all slides from that test once, but reading additional tests from the same sample is not necessary.

### Keeping Records

Maintaining accurate records of samples is extremely important. Always record information in ink. **NEVER** use correction fluid on laboratory documents, slide labels, or sample labels. If a correction is necessary, draw a line through the incorrect entry, enter the correct entry, and place your initials and date beside the correct entry.

### Tilletia indica Microscopy Log Sheet:

Keep your own record of the samples and their counts using the *Tilletia indica* Microscopy Log Sheet (see **Appendix 1** for an example). Record the sample #, set # (box #), number of spores, and 1st/2nd reads. Use the Sample # section to record slide number, if there are multiple slides. For example, “1 of 2 slides.” Record spore coordinates in the Comments section. Each reader should do an equal number of 1st and 2nd reads during a shift. Submit the *Tilletia indica* Microscopy Log Sheets to the appropriate person at the end of your shift.

### Slide Labels:

After completing a slide, place your initials **and** the date on the slide label.

**Tilletia indica Spore Analysis Worksheet:**

Return the slide to the appropriate box. Record the following data on the TILLETIA INDICA SPORE ANALYSIS WORKSHEET (see **Appendix 1** for an example):

- Number of spores counted
- Your initials
- The date

Have the verifier who confirmed the first *T. indica* teliospore record his or her initials under the Confirmed By section. In the comments section, record information such as “estimated” if the count is not actual.

**Interpreting  
Laboratory  
Results**

A laboratory result, whether positive or negative, stands on its own. Repeating a test does not necessarily invalidate or validate the previous test unless the results are the same. If a negative follows a positive test, as with harvest samples, running two additional tests will be necessary.

**Rating on the Karnal Bunt Spore Analysis Worksheet:**

After a slide reader reads the slides and documents the results on the worksheet, the laboratory manager will rate the results using the following codes (only one rating is necessary per sample):

- **P** for **positive**. Used when at least one spore of *T. indica* is confirmed for the sample.
- **N** for **negative**. Used when no *T. indica* spores are confirmed for the sample.
- **X** for **retest**. Used when the verifier cannot make a positive identification.
- **L** for **laboratory accident**. Used when a sample is spilled, mislabeled, etc. Retesting is required.
- **R** for **reject**. Used when there are multiple test results for the same sample. One test is rated and the others are rated with an R.

## LABORATORY PROCEDURES

### Examining Wheat Kernels for Karnal Bunt

#### Introduction

This subsection describes procedures used to identify wheat kernels infected with *Tilletia indica*, the causal agent of Karnal bunt (KB). These infected kernels are called bunted kernels. Examine all samples, positive and negative, for bunted kernels following completion of extraction and microscopic analysis. Included are specific procedures for decontamination of samples and facilities. The area designated for this work must be physically separate from the area where spore analyses are done.

#### Supplies

Laboratory personnel will need the following reagents, equipment, and other items to examine wheat kernels for KB:

##### Reagents:

- Bleach, household, 5.25 percent solution
- Shear's mounting fluid (see **Extracting Samples** subsection for preparation)

##### Suggested Equipment and Other Items:

- Bags, biohazard, 12" x 24"
- Beaker, glass, 600-mL
- Bottle, spray, 1-L, hardware store
- Boxes, cardboard
- Caps, hair
- Coveralls, L, XL, and XXL
- Covers, shoe
- Coverslips, glass, 22 x 60 mm
- Dish, petri, plastic disposable, 50 x 9 mm
- Dish pans, plastic, department store
- Forceps, specimen, stainless steel, large tip
- Garden hose with nozzle, hardware store
- Gloves, latex, medium and large
- Hood
- Labels, white paper
- Lamp, magnifying
- Microscope, dissecting
- Slides, microscope, 3" x 1"
- Storage units
- Timer
- Towels, lab
- Vials, plastic
- Wash basin, double sided
- Worksheets, Karnal Bunt Kernel Analysis, in-house

**Obtaining  
Samples**

The laboratory manager will provide sample numbers for examination. Examine positive samples (lots that come up positive for *T. indica* teliospores, as listed on an analysis worksheet) on a separate day or shift from negative samples. This precaution is to help prevent cross contamination of the samples and to allow for proper decontamination of the bunt area.

Box # and Sample # identify samples. The box # should correspond to cardboard boxes stored in the storage units, with each box containing four or more samples in bags labeled with the Sample #. Return each sample or box of samples to its original location in the storage unit following examination. Keep the unexamined samples at one end of the bunt area, and as they are finished move them to the other end.

The bunt area is **off limits** to untested field samples and field samplers. If assigned the task of detecting bunted kernels, **do not enter any other KB analysis areas**. Always wear coveralls, along with disposable booties and caps. When examining seed, wear disposable surgical gloves. Remove these articles when leaving the bunt area.

**Preparing the  
Workstation**

Designated bunted kernel readers must wear clothing that will prevent sample contamination and protect them from harmful chemicals.

**Step 1:**

Put on your shoe covers, hair cap, coveralls, and latex gloves. If the seed samples show pink or violet dye, wear a dust mask when examining the seed. The dye shows that the seed has been treated with fungicide.

**Step 2:**

Take one 600-mL beaker, one petri dish, one large forceps, and one 11" x 14" white plastic dish pan (tray) from the biosafety hood or clean air station. Set these items up at your workstation and use the TILLETIA INDICA SPORE ANALYSIS WORKSHEET for recording data.

**Step 3:**

Turn the hood on. From the sealable plastic bag holding the seed sample, scoop approximately 500 mL of seed and pour it into the beaker. Take the sample to your workstation for examination.

**Step 4:**

Spread half the sample (250 mL) throughout the plastic tray in approximately one layer of seed. Begin by doing a visual overview of the sample in the tray, looking for seed that shows a darkening on one end or obvious deterioration of the seed tissue.

**Identifying  
*Tilletia indica***

Early stages of infection first appear at the germ end of the wheat kernel. These symptoms are usually not visible when the groove (dorsal) side is facing down. Therefore, an effective technique is to turn many seeds over to look for symptoms.

The first visible stage of KB infection is a swollen area that appears gray or dark at the germ end forming a triangular shaped area on either side of the groove (see **Appendix 1, Symptoms of Karnal Bunt (*Tilletia indica*) and Black Point, A2, B1 & 2**). This is the thin pericarp, or outer epidermal layer, of the seed coat through which a darkening is visible and is evidence of a mass of teliospores beneath (the sorus). This swollen area of the pericarp may already be ruptured or can easily be punctured with a teasing needle, exposing many dark brown teliospores.

KB teliospores, large by fungal spore standards, are easy to see with a hand lens. However, to verify KB symptoms from a seed sample, viewing the infected kernels under a dissecting microscope will be necessary to see if teliospores are present. They appear as hundreds or thousands of small, granulate, shiny dark brown to black spheres, resembling microscopic ball bearings or buckshot. They are contained within the area of the seed that the fungus has consumed, together forming the sorus.

More advanced infection (**A3 & 4**) in the wheat kernel manifests itself as a blackening along the groove, progressively consuming more of the inner portions of seed tissue. A very advanced stage of infection results in the wheat kernel taking on the appearance of a boat or dugout canoe (**A5**).

Black point is a disease of non-quarantine importance caused by a variety of fungal species. The superficial symptoms of KB have been confused with those of black point, but close examination reveals distinct differences. While both diseases give the germ end of the wheat a darkened appearance, black point is actually more of a darkening or discoloration of the pericarp (**C1 & 2**). Upon examination under a dissecting microscope, no spores, nor anything that resembles teliospores, are visible. Also, the darkening caused by black point organisms may extend farther from the tip of the seed and be more general in its occurrence on the seed, often appearing on the ventral and dorsal surfaces. Black point shows no obvious erosion of the seed tissue as in KB.

Infections by other wheat smut species may be present, but none have the characteristic appearance of KB teliospores when viewed under a dissecting microscope. These other smut species have much smaller spores that often fill the seed before it ruptures, and have a more grayish, dusty appearance to the spore mass.

**To make a positive identification for any spore, making a microscope slide will be necessary to view the spores under high magnification.**

**Storing Positive  
Kernels****Step 1:**

Place all of the bunted kernels from the same sample in a plastic vial. Using a black marker, label the vial with the sample number on the outside of the vial, and inside the vial on a white label.

**Step 2:**

Get an identifier to verify the presence of *T. indica* on the kernels found in a sample. Return all negative seed to its original packaging. Also return the positive sample (minus any bunted kernels) to its original packaging and place it in the storage area.

**Step 3:**

Record your findings, initials, and date on the KARNAL BUNT KERNEL ANALYSIS WORKSHEET (see **Appendix 1** for an example). Have the verifier initial in the appropriate space for the positive samples verified.

**Step 4:**

Place all labware and instruments into the dirty bin for decontamination. Use new gloves, labware, and instruments for each new sample. Continue with the next sample.

**Decontaminat-  
ing Labware  
and  
Instruments**

The dishwasher will take all dirty labware and instruments to sinks in a designated area for decontamination. Soak all labware and instruments in a 30 percent bleach solution for 15 minutes, rinse with clear water, dry with lab towels, and return to the bunt area for the next use.

After examining all the samples, or at the end of a shift, decontaminate the area. Decontamination includes these activities:

- Bleach all workstation surfaces (hoods, chairs, tables, etc.)
- Make sure no dirty labware or instruments remain in the trailer
- Sweep the floor and mop with a mop soaked in 30 percent bleach solution

Before bringing in negative samples, remove all samples microscopically identified as positive. When decontamination is complete, dispose of all shoe covers, hair covers, coveralls, and gloves into the biohazard container. Dispose of all waste during the examination process into the biohazard container. Turn off all hoods and lights when finished. Be sure to turn the hoods back on 15–20 minutes before resuming examination activities at the beginning of the next day.

**Please handle all samples as if they are positive to help prevent contamination.**

## LABORATORY PROCEDURES

### Training Readers and Verifiers

#### Introduction

To assure accuracy and efficiency when laboratory personnel examine slides, slide readers need to go through a period of training with practice slides before they start reading real samples. Verifiers, as well, should have training in examining isolates of *Tilletia indica*, and should meet certain educational and experience requirements. The following training recommendations were developed by Walter Pearson, retired PPQ Plant Pathology Identifier, Laredo, Texas, and Joel Floyd, Supervisory PPQ Officer/Plant Disease Identifier, Nogales, Arizona.

#### Training Readers

##### Recommended training content and guidelines follow:

1. Assume that the trainees know nothing about Karnal bunt and smuts.
2. Discuss what a smut is and how it affects the plant. Show and discuss the life cycle of *T. indica*.
3. Show the identifying characters of *T. indica*:
  - Size (compare with *T. barclayana*)
  - Pattern on spore surface
  - Truncate ornamentations
  - Variation in spore color and shape
  - Sheath
4. Discuss how other species of *Tilletia* compare with *T. indica* (spore sizes, pattern on exospore, ornamentation, color, sheath)

##### Recommended training procedures follow:

1. Ensure that trainees know how to use the microscope and the ocular micrometer.
2. Have trainees study *T. indica* reference slides, noting immature spores, sterile cells, and variations in shape and color of mature spores.
3. Work with trainees on training scope:
  - Note dirt particles, pollen grains, other *Tilletia* species spores, bleached spores, immature *T. indica* spores, and spore fragments
  - Point out the characters that separate the “look alike” from *T. indica*
4. Discuss **Appendix 3, Estimating the Number of Teliospores of *Tilletia indica* in Heavily Infested Samples.**

5. Ensure that trainees can accurately measure spore diameters with the ocular micrometer.
6. Have trainees read 25 training slides for an accurate spore count. Ask trainees to reread slides with inaccurate spore counts, especially the false negatives.
7. Teach paper work procedures, make sure trainees understand the work protocol, then “graduate” the trainees.

### Selecting and Training Verifiers

The Karnal bunt plant pathology verifier must be able to positively identify *Tilletia indica* Mitra, the causal agent of Karnal bunt, to the exclusion of other fungi. The verifier serves as the final authority for identification of KB.

#### Requirements:

The verifier should hold a Master's or Doctoral Degree in mycology, plant pathology, or other related fields. The verifier should have work experience with microscopy, fungal taxonomy, and morphology, or extensive course work in plant pathology and mycology and work related experience in plant pathology and mycology. Knowledge or experience in plant pathology is desirable but not a requirement. For those without a graduate degree in plant pathology or a related field, the requirement should be a thorough foundation in mycology or plant pathology with recent work experience in that area.

#### Job Description (the incumbent is responsible for these duties):

1. Verifies the identification of *T. indica* teliospores found by microscopists during KB screening to the exclusion of other fungi.
2. Distinguishes by microscopic examination other species of *Tilletia* frequently encountered (for example, *T. fusca* and *T. eragrostidis*).
3. Using established guidelines on spore size, makes determinations of when to send “suspect spores” to National Mycologist for final determination.
4. Confirms the identification of wheat kernels infected by *T. indica*.
5. Maintains familiarity with current information on the *Tilletia* pathogen infecting ryegrass.

### Training:

Training will consist of microscopic examination of several isolates of *T. indica*. Depending on their age and condition, teliospores will exhibit significant variation in size, shape, and color. Verifiers will frequently find immature and fragmented spores.

KB Plant Pathology Verifiers will complete the following training activities:

- Analyze slides with mixtures of *T. indica* and *T. barclayana* (rice smut). This exercise will be particularly valuable to verifiers working in California and the Gulf States due to frequent contamination of grain handling facilities and conveyances with rice smut.
- Review microscope slides of actual samples to familiarize themselves with the other fungi commonly found associated with wheat and pollen grains frequently encountered.
- Scan a minimum of 10 training slides of actual wheat samples, with known numbers of teliospores. At least one of these slides will be a negative control. The purpose of this training slide exercise is to familiarize the verifiers with the microscopist's tasks, not for them to become skilled microscopists. For the verifier to qualify as a reader, the verifier must meet the requirements for readers described in the previous subsection, **Training Readers**.
- Examine wheat kernels macroscopically and learn how to positively confirm a bunted kernel caused by *T. indica*.

### Retesting:

A system of retesting verifiers should be in place. This should include examining slides with various wheat smuts, particularly variations of *Tilletia* species.

### Discard Authority:

After the candidate completes the required training and shows the ability to identify *T. indica* teliospores and bunted kernels in KB Project samples, designated USDA personnel will grant Discard Authority for use on the KB Eradication Project.







## APPENDIX 1

### Examples of Forms and Identification Aids

#### Introduction

This appendix provides examples of completed forms and blank forms used in the KB Emergency Program. Also included are aids to help identify KB teliospores and bunted wheat kernels.

Listed below are the contents of this appendix:

- Example of a Completed *Tilletia indica* Spore Analysis Worksheet
- Blank *Tilletia indica* Spore Analysis Worksheet
- Example of a Completed *Tilletia indica* Microscopy Log Sheet
- Blank *Tilletia indica* Microscopy Log Sheet
- Example of a Completed Karnal Bunt Kernel Analysis Worksheet
- Blank Karnal Bunt Kernel Analysis Worksheet
- Karnal Bunt Teliospores, *Tilletia indica* Mitra
- Symptoms of Karnal Bunt (*Tilletia indica*) and Black Point

TILLETIA INDICA SPORE ANALYSIS WORKSHEET

Set 1649

SAMPLE #	RATING	Slide Taken By	Slide	# of Spores	ID By	Date	Confirmed By	Comments	Data Entered
KB96-6491	N	D LC	1/1	0	LC	7-18-96	DR	1 slide	LC
KB96-6492	N	D <del>LC</del> KRH	1/1	0	KRH	7-18-96	LC	1 slide	
KB96-6493	N	D Lms	1/2	0	Lms	7-18-96	VP	2 slides	
		D WDM	2/2	0	WDM	7-18-96	LC		
KB96-6494	N	D DR	1/1	0	DR	7-18-96	WDM	1 slide	
KB96-6495	P	D Lms	1/1	0	Lms	7-18-96	WDM	1 slide	
KB96-6496	N	D KRH	1/1	0	KRH	7-18-96	DR	1 slide	

DATA ENTRY CONFIRMED BY (NAME/DATE):

Anna V. Ponder / 7-18-96

*Figure 5-1: Example of a Completed Tilletia indica Spore Analysis Worksheet*

DATA ENTRY CONFIRMED BY (NAME/DATE):

**Figure 5-2: Blank *Tilletia indica* Spore Analysis Worksheet**



## Identifier: \_\_\_\_\_

Date: \_\_\_\_\_

Shift: \_\_\_\_\_

[illegible]

*Figure 5-4: Blank Tilletia indica Microscopy Log Sheet*

## KARNAL BUNT KERNEL ANALYSIS WORKSHEET

Positive BOX #	SAMPLE #	KERNELS			CONFIRMED BY	COMMENTS	DATA ENTERED
		#	IDENTIFIED BY	DATE			
201	KB96-7257	φ	Rm 1+	10-5-96			PK 10/5/96
201	KB96-7272	φ	PSS	10-5-96			
201	KB96-7281	φ	Rm 1+	10-5-96			
201	KB96-7284	φ	W V	10/5/96			
209	KB96-7550	N/A		10/5/96		No Sample... Bag Empty	
210	KB96-7578	φ	PSS	10-5-96			
211	KB96-7588	φ	W V	10/5/96			
216	KB96-7602	φ	PSS	10-5-96			
213	KB96-7615	φ	Rm 1+	10-5-96			
216	KB96-7636	φ	PSS	10-5-96			
218	KB96-7647	φ	Rm 1+	10-5-96			
218	KB96-7653	φ	W V	10/5/96			✓

DATA ENTRY CONFIRMED BY (NAME/DATE):

Paul S. Knapp 10/5/96

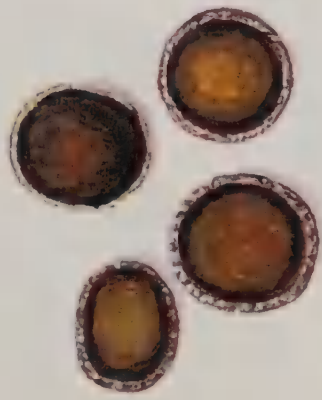
Figure 5-5: Example of a Completed Karnal Bunt Kernel Analysis Worksheet

[illegible]

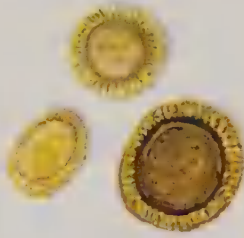
DATA ENTRY CONFIRMED BY (NAME/DATE):

**Figure 5-6: Blank Karnal Bunt Kernel Analysis Worksheet**

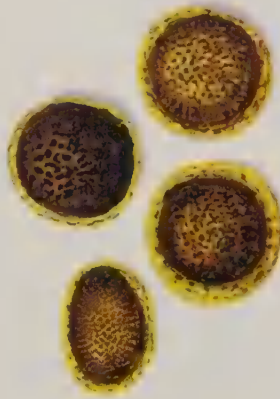




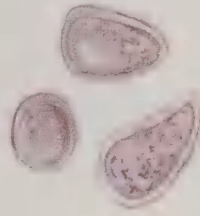
medial cross-  
sectional  
focusing  
plane



Immature teliospores



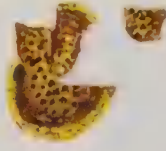
top view,  
tangential  
focusing  
plane



Sterile spores



Bleached teliospores



Broken teliospore

Mature teliospores

size range: 24 - 47 $\mu$  ave. 39 $\mu$

# Karnal Bunt Teliospores, *Tilletia indica* Mitra

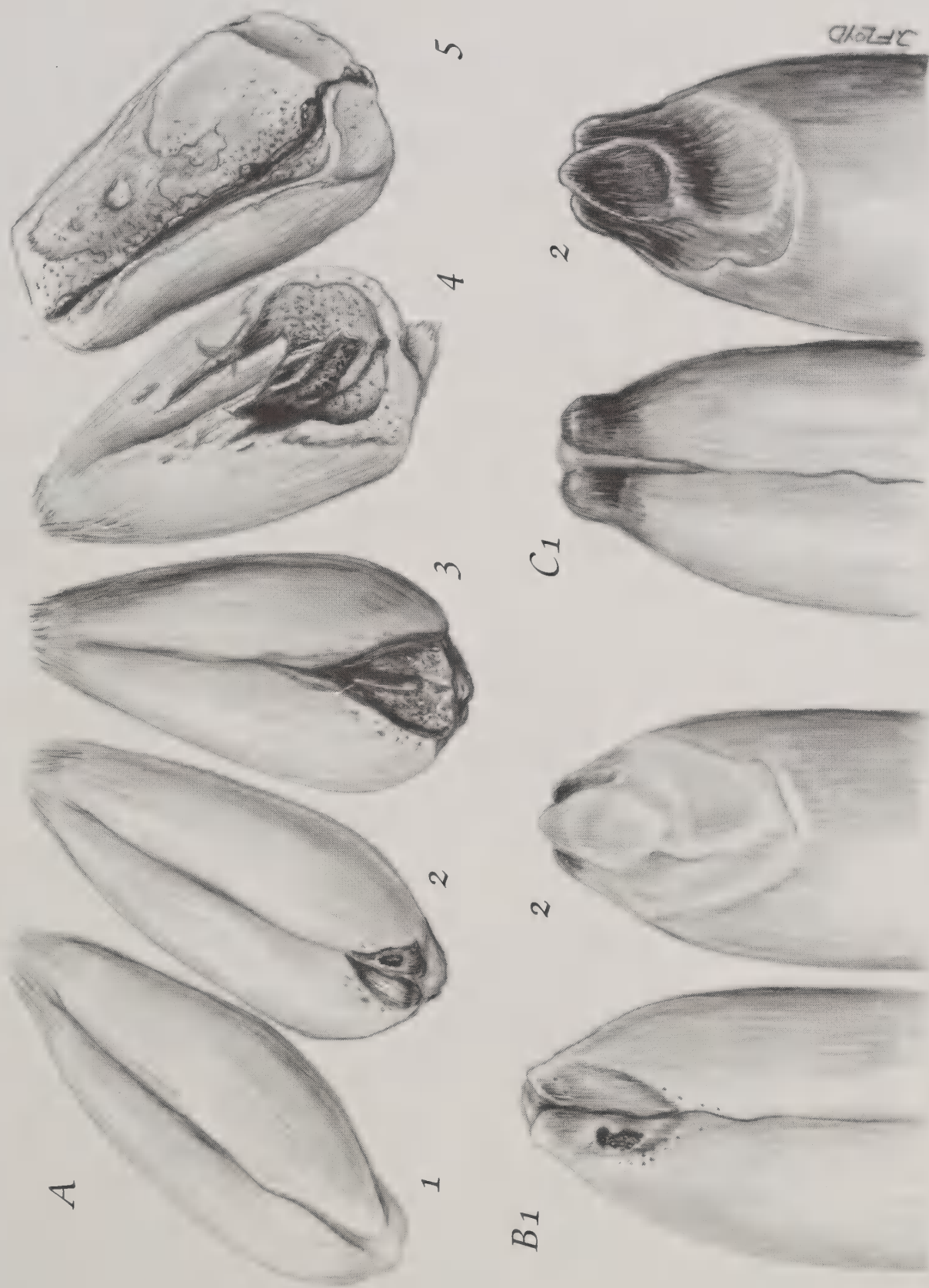
*Stages of teliospore development and variations in appearance as viewed on a compound microscope slide.*

WILLIAM BROWN, JR.

1871

1871

1871



Symptoms of Karnal Bunt (*Tilletia indica*) and Black Point. A1, Healthy wheat kernel; A2, early symptoms of *T. indica* infection; A3 & A4, progressively advanced *T. indica* infection; A5, advanced infection of *T. indica* with "canoe" shaped kernel. B1, early stage of *T. indica* on dorsal side of wheat kernel with ruptured pericarp and teliospores forming sorus beneath; B2, ventral surface of *T. indica* infection; C1 & C2, Black Point symptoms, dorsal and ventral respectively.



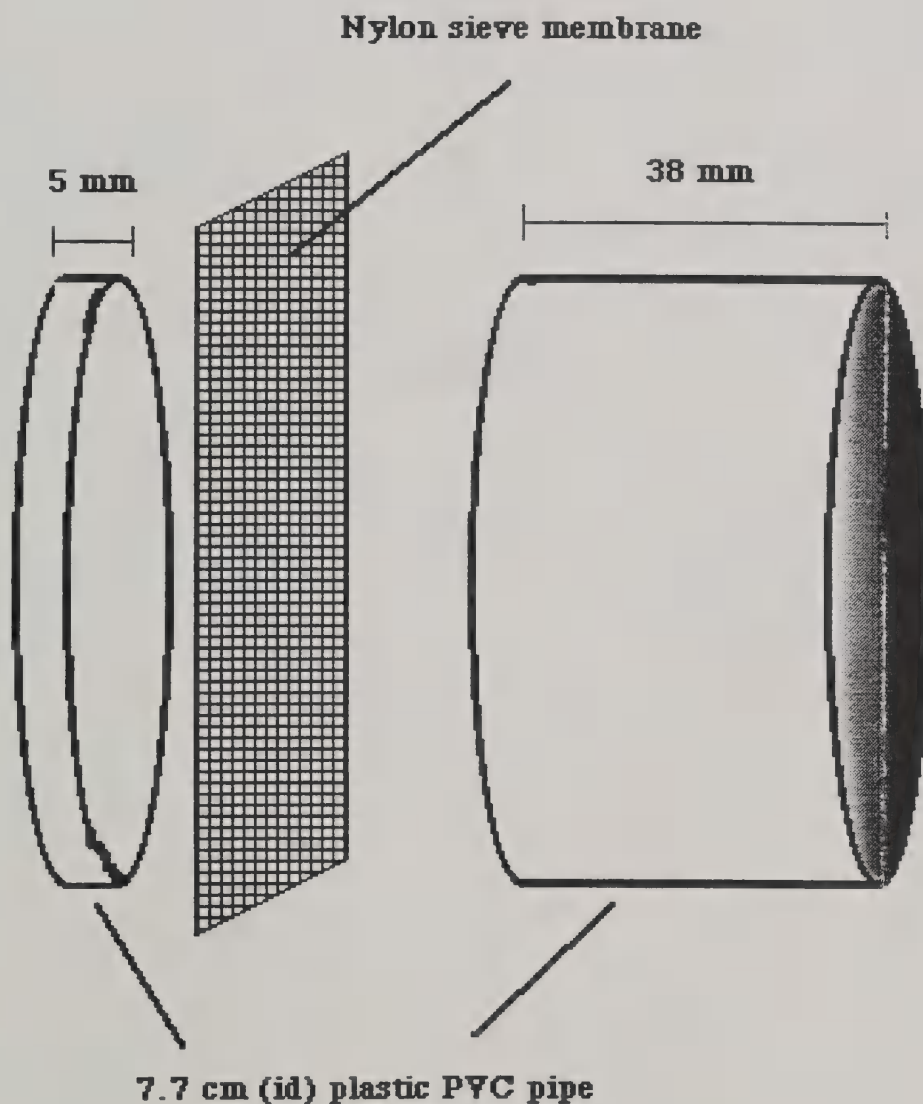




## APPENDIX 2

### Construction of Nylon Sieves

Illustrated below is a drawing showing how to construct the sieves. Assemble with PVC pipe cement; apply a heavy coat on both pipe surfaces in contact with the nylon sieve.









## APPENDIX 3

Estimating the Number of *Tilletia indica* Teliospores in Heavily Infested Samples (technique developed by Gary L. Peterson, USDA-ARS)

1. For each microscope, determine the number of optical fields across the width and length of the coverslip.

For example, 7 fields from front to back = W  
 18 fields from left to right = L  
 $7 \times 18 = 126$  fields per slide = T

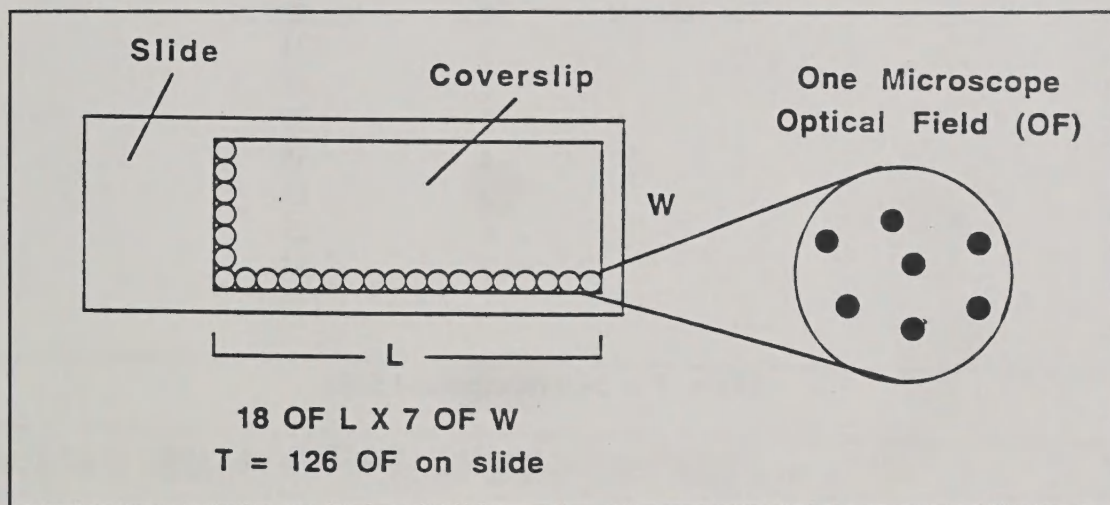


Figure 7-1: Optical Fields Across Width and Length of a Coverslip

2. If the teliospore density is between 3 and 20 spores per optical field:
  - a. Count the number of teliospores in each of three randomly selected\* passes across the length of the slide and determine the average (Figure 7-2A).
  - b. Multiply the number of optical fields from front to back (W) by the average number of teliospores in a pass. Report this number.

For example,

<u>pass</u>	<u>no. spores</u>
1	70
2	118
3	85
	<hr style="width: 50px; margin: 0;"/> 273

$273 \div 3 = 91$  teliospores

$91 \text{ teliospores} \times 7 (W) = 637 \text{ teliospores on slide}$

- c. If the entire sample suspension was not on the slide examined, divide the total number of teliospores estimated on the slide by the number of drops applied to the slide and multiply this number by the total number of drops in the sample. Report this number.

3. If the teliospore density is greater than 20 spores per optical field:
  - a. Determine the average number of teliospores in seven randomly selected\* fields (*Figure 7-2B*).
  - b. Multiply this number by the total number of optical fields (T) on the slide. Report this number.

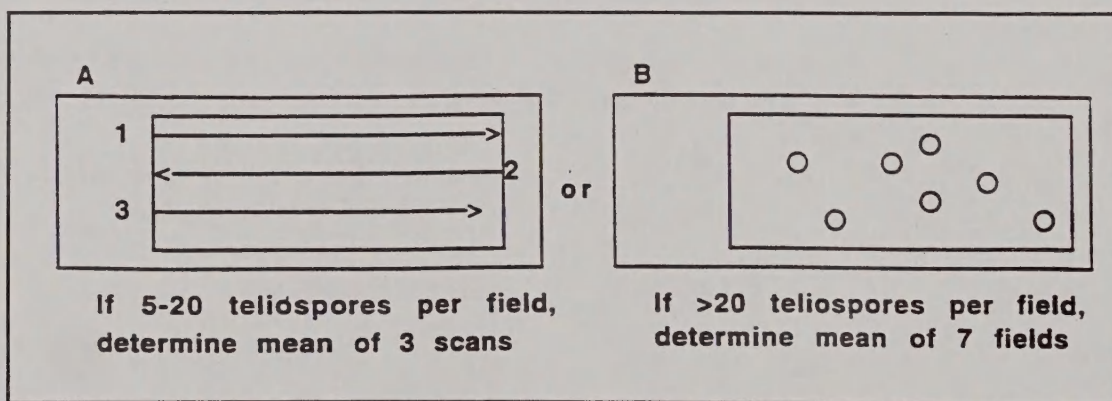
For example,

<u>field</u>	<u>no. spores</u>
1	21
2	32
3	27
4	38
5	15
6	23
7	18
	<hr/> 174

$$174 \div 7 = 24.9 \text{ teliospores / field}$$

$$(24.9 \text{ teliospores / field}) \times (126 \text{ (T) fields / slide}) = 3,137 \text{ spores}$$

- c. If the entire sample suspension was not on the slide examined, divide the total number of teliospores estimated on the slide by the number of drops applied to the slide and multiply this number by the total number of drops in the sample. Report this number.



*Figure 7-2: A, Randomly Selected Passes, and B, Randomly Selected Fields on a Microscope Slide*

\* Random selections are made by moving the optical field of view to a position on the slide without looking through the oculars. Do not overlap a previously selected field or scan.

**KARNAL BUNT EMERGENCY PROGRAM MANUAL**  
**Comment Sheet**

Directions: Use this sheet to suggest an improvement or to identify a problem in the content of the manual. To mail, please follow the directions on the next page.

Description of problem (error, inconsistency, missing or insufficient information, etc.):

Description of improvement or recommended change (add attachments if necessary):

=====  
Reason for improvement or change:

AFTER COMPLETION, FOLD ON THE DOTTED LINES WITH THE ADDRESS SIDE OUTWARD.  
STAPLE OR TAPE TO CLOSE, AFFIX POSTAGE, AND DROP IN THE MAIL.

USDA-APHIS-PPQ  
7340 Executive Way, Suite A  
Frederick, MD 21704

Attn: Bruce Attavian